CERTIFICATE OF FACSIMILE TRANSMISSION

I hereby certify that this correspondence is being transmitted to the U.S. Patent & Trademark Office in accordance with 37 CFR § 1.6(d) on the date indicated.

Name Date June 29, 2004

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventors: Choy-Pik Chiu & Robert Kay

Filing Date: November 21, 2001

Serial No: 09/990,522

Docket: 097/002

Title: TOLERIZING ALLOGRAFTS OF PLURIPOTENT STEM CELLS

Art Unit: 1636

Examiner: Quang Nguyen, Ph.D.

SECOND DECLARATION UNDER 37 CFR § 1.132
BY ANISH S. MAJUMDAR, Ph.D.

Commissioner for Patents
Alexandria VA 22313

Dear Sir:

I, ANISH MAJUMDAR, do hereby declare as follows:

I am Director of Immunology at Geron Corporation. I have provided a previous Declaration under § 1.132 in support of the application referred to above. This second Declaration provides additional information for consideration by the Patent Office.

My previous Declaration provided data showing:

- undifferentiated hES cell lines are unable to induce allo-reactivity in primary human T cells
- hES cells actively suppress immunoreactivity, even between stimulators and responders that are otherwise highly immunogenic
- neural progenitor cells made from hES cells also f ail to induce significant T cell proliferation by allogeneic responder cells in vitro.

I understand the Examiner has questioned whether hES derived cells of the mesenchymal lineage also retain the toleragenic properties of the undifferentiated hES cells, and can therefore be used to induce tissue tolerance as described in the patent application.

Attached to this Declaration are results of some additional experiments.

These experiments were done with a cell line derived from hES cells that bears markers shown to be expressed on mesenchymal stem cells, and has been immortalized by transducing with the gene for telomerase reverse transcriptase (hTERT).

Briefly, hES cells from the H1 line were harvested, dissociated into small clusters, and cultured in non-adherent cell culture plates to form aggregates. After 4 days in suspension, the aggregates were transferred into gelatin-coated plates and cultured for an additional 9 days. The outgrowth culture were serially passaged, and after two passages, the cell population appeared homogeneous with morphological characteristics of fibroblasts. The cell line was designated **HEF1.**

Subpopulations were transduced with a retrovirus telomerase expression vector (pBABE-hTERT) (Ouellette et al., Hu. Mol. Gen. 9:403, 2000), and selected using puromycin. The replicative capacity of the original HEF1 line was about 12 doublings, whereas the hTERT transduced cells replicated for over 30 doublings while retaining the same characteristics.

Figure 1 shows marker expression determined by flow cytometry. The HEF1 cells were compared with human mesenchymal stem cells (hMSC) obtained from Clonetics Corp., San Diego CA. Specific staining is shown by the open line, while solid areas show the isotype control.

The HEF1 cells expressed CD29, CD44, CD71, and CD90 at similar levels as the hMSC. Neither CD45 nor CD14 was observed in either cell line.

Figure 2 shows what happened when cells were treated with osteogenic factors (100nM dexamethasone, 50 μM ascorbic acid, and 10 mM β-glycerophosphate). Both the HEF1 cells and hMSC differentiated into distinct nodules that stained with Alizarin red, and showed increased calcium deposition and alkaline phosphatase activity. These are features of osteoblast lineage cells. In contrast, HEK293 cells, a non-osteogenic cell line used as negative control, failed to respond to the same conditions.

Thus, the HEF1 cells shared with classic hMSC cells not only characteristic cell surface markers, but the ability to differentiate further into cells having functional activity of osteoblasts. We have not yet shown that HEF1 cells are capable of generating adipocytes or chondrocytes.

Figure 3 shows levels of HLA expression. HEF1 cells express HLA Class I antigens. Neither HEF1 cells nor hES cells express HLA Class II antigens.

Figure 4 is taken from an experiment in which HEF1 cells were found to inhibit an immune reaction by third-party cells.

Peripheral blood leukocytes were isolated from two normal human donors on a density gradient. To enrich for T lymphocytes, separated cells were incubated for 2 h at 37°C, and the non-adherent cells were collected. Dendritic cells (DCs) were prepared by culturing the remaining adherent cells for 7 days in AIM V medium containing human recombinant GM-CSF and IL-4.

The DCs induce significant proliferation of T cells from the other (allogeneic) donor, but not from the same (autologous) donor from which they were isolated.

Undifferentiated hES cells of the H1 line and the H9 line, HEF1 cells, and BJ fibroblasts were titrated into parallel allogeneic MLRs to determine what effect they had on the immune reaction between the donor cells. In some experiments like the one shown here, the HEF1 cells actively inhibited the ability of the third-party donor DCs to stimulate the allogeneic T cells. This inhibitory capacity is a special property of these cells, which it shares with undifferentiated hES cells but not with other cell types like BJ fibroblasts.

These results show that cells differentiated from hES cells and expressing characteristic mesenchymal stem cell markers retain the toleragenic properties of undifferentiated hES cells — including the ability to inhibit immune stimulation between third party cells.

This supports the use of hES derived mesenchymal cells (like HEF1 cells) for inducing immunotolerance in the same manner as the invention in this patent application.

8. I hereby declare that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

6-28-04
Date

A. Sen Majumdar
Anish Sen Majumdar, Ph.D.
Menlo Park, California

Figure 1

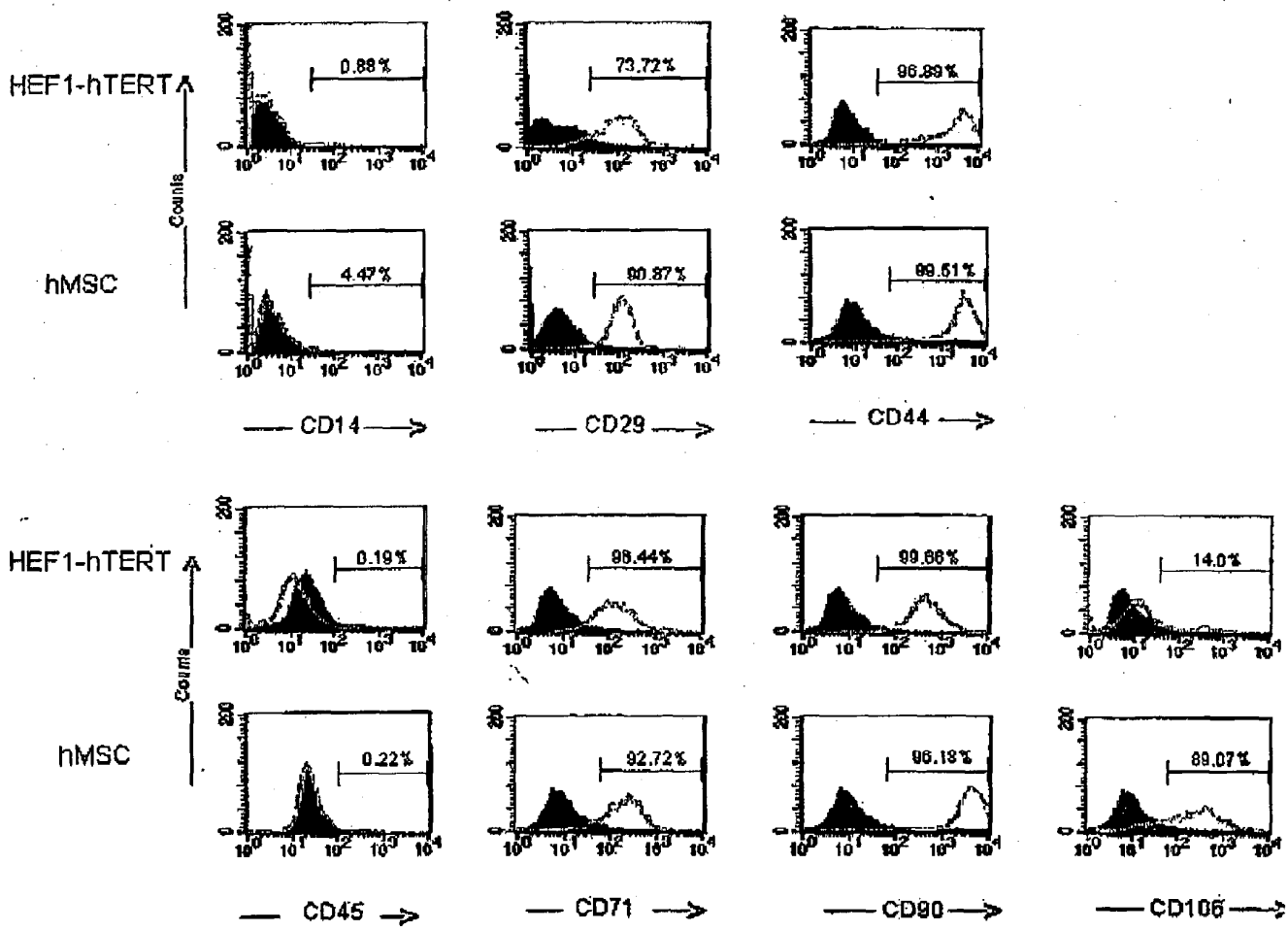
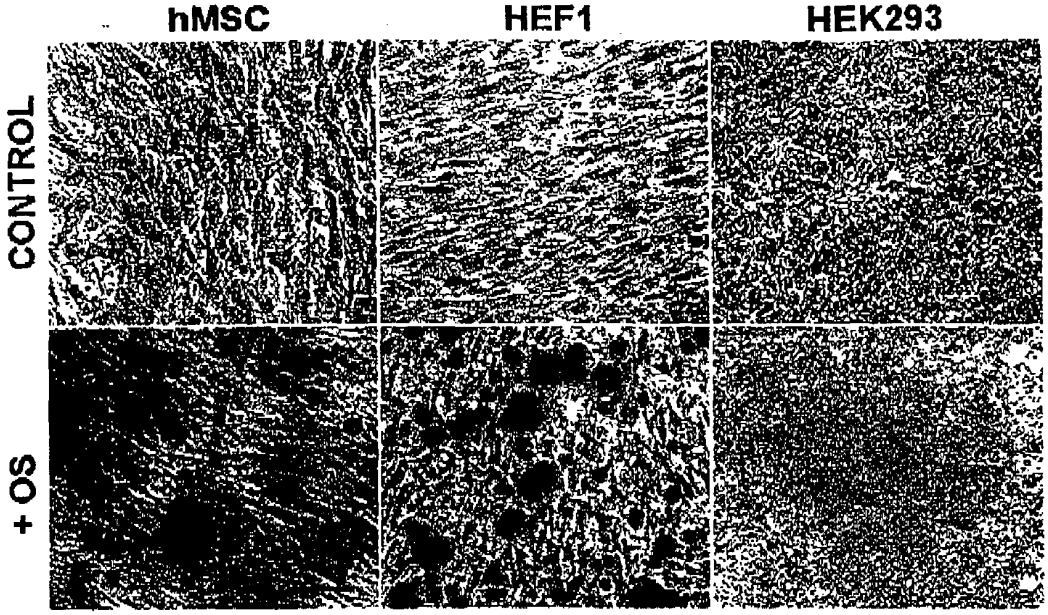
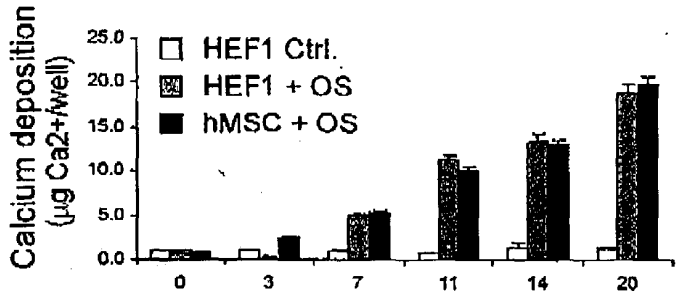

HEF1-hTERT
hMSC
Counts
0.88%
73.72%
96.89%
4.47%
80.87%
99.61%
CD14
CD29
CD44
HEF1-hTERT
hMSC
Counts
0.19%
98.44%
99.66%
14.0%
0.22%
92.72%
96.18%
89.07%
CD45
CD71
CD90
CD106

Figure 2


hMSC
HEF1
HEK293
CONTROL
+ OS


Calcium deposition
(µg Ca2+/well)
25.0
20.0
15.0
10.0
5.0
0.0
HEF1 Ctrl.
HEF1 + OS
hMSC + OS
0
3
7
11
14
20

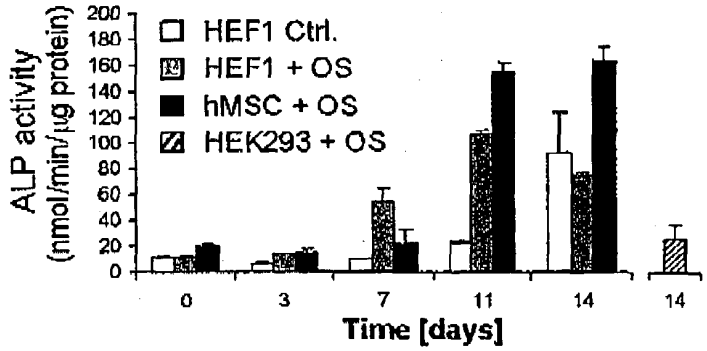

ALP activity
(nmol/min/µg protein)
200
180
160
140
120
100
80
60
40
20
0
HEF1 Ctrl.
HEF1 + OS
hMSC + OS
HEK293 + OS
0
3
7
11
14
14
Time [days]

Figure 3

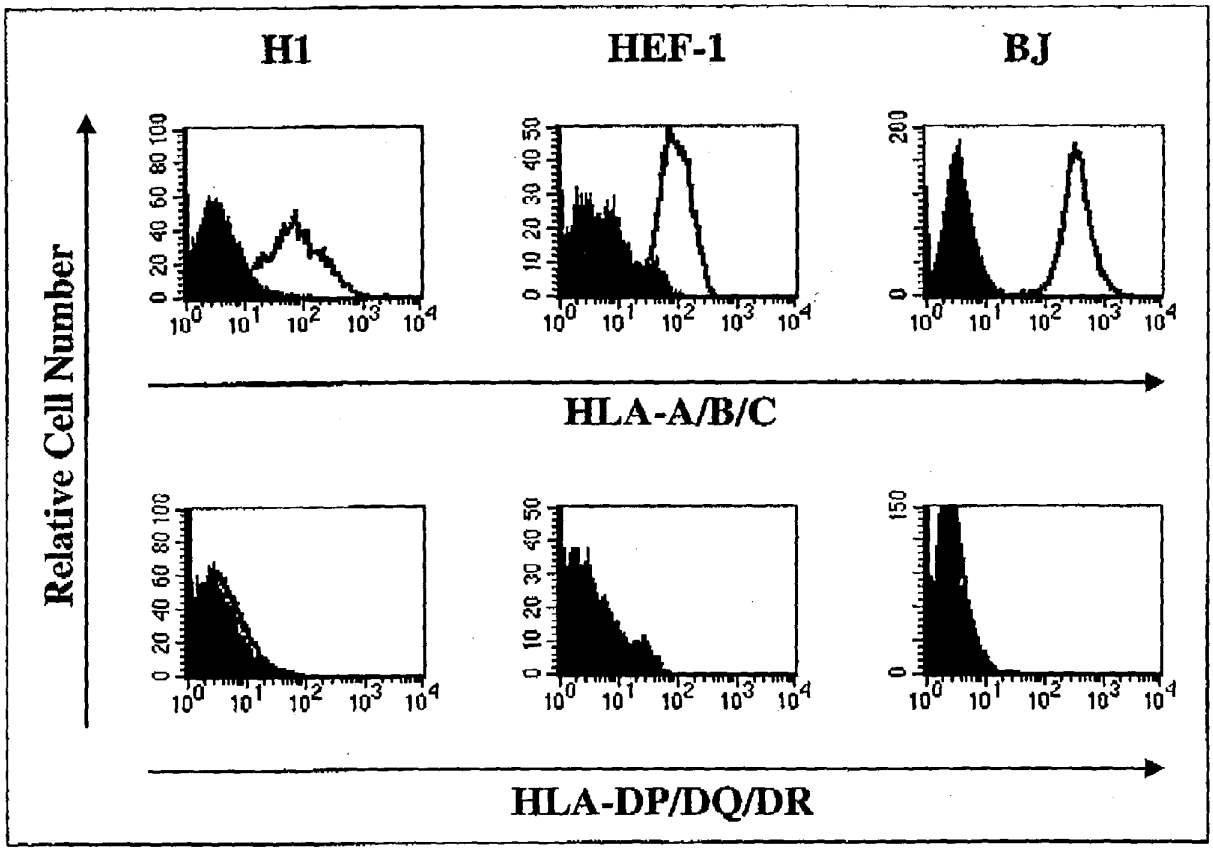
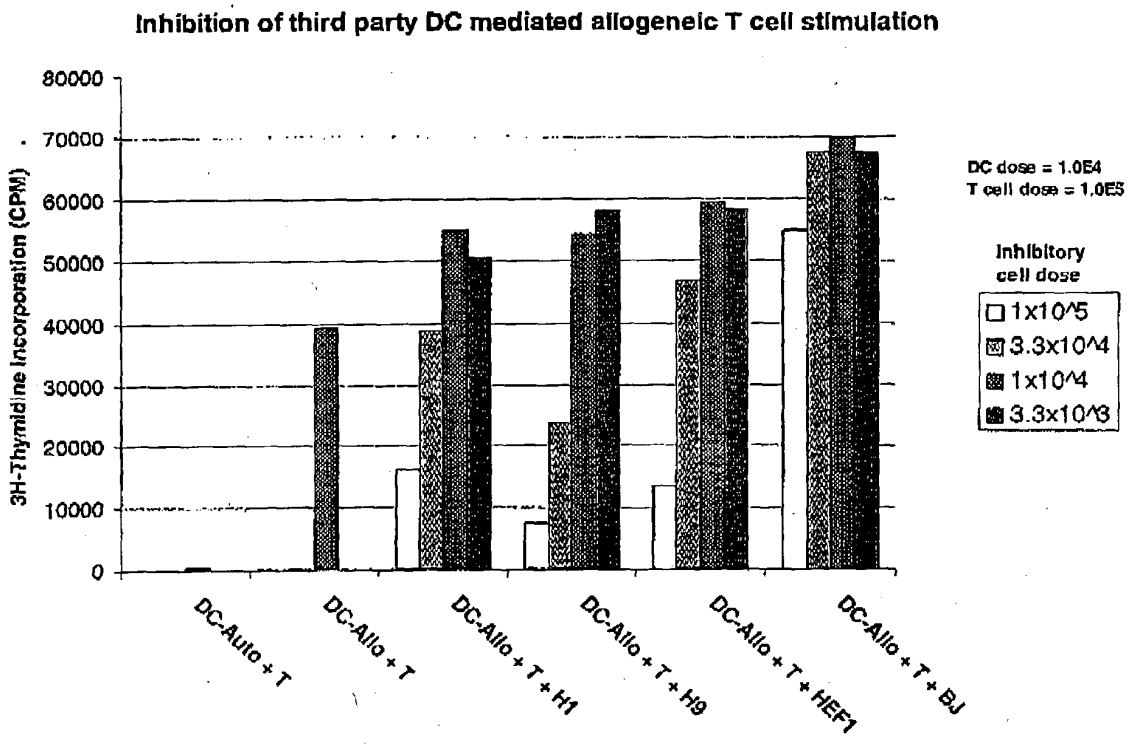


Figure 4


Inhibition of third party DC mediated allogeneic T cell stimulation
3H-Thymidine Incorporation (CPM)
80000
70000
60000
50000
40000
30000
20000
10000
0
DC-Autlo + T
DC-Allo + T
DC-Allo + T + H1
DC-Allo + T + H9
DC-Allo + T + HEF1
DC-Allo + T + BJ
DC dose = 1.0E4
T cell dose = 1.0E5
Inhibitory cell dose
1x10^5
3.3x10^4
1x10^4
3.3x10^3